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An Interleukin-2 Signal Relieves BSAP (Pax5)-Mediated Repression of the Immunoglobulin J Chain Gene

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Summary

Cytokine regulation of B cell development was analyzed using interleukin-2 (IL-2)-induced transcription of the J chain gene as a model system. A nuclear target of the IL-2 signal was identified as the *Pax5* transcription factor, BSAP, which recognizes a negative regulatory motif in the J chain promoter. Functional assays showed that BSAP mediates the silencing of the J chain gene during the early stages of B cell development, but repression is relieved during the antigen-driven stages in a concentration-dependent manner by an IL-2-induced down-regulation of BSAP RNA expression. At the low levels present in J chain–expressing plasma cells, BSAP repression could be overridden by positive-acting factors binding to downstream J chain promoter elements. Overexpression of BSAP in these cells reversed the positive regulation and inhibited J chain gene transcription. Thus, IL-2 regulation of BSAP concentration may provide a mechanism for controlling both repressor and activator functions of BSAP during a B cell immune response.

Introduction

The J chain gene is one of the few identified nuclear targets of interleukin-2 (IL-2) signaling. Its expression is initiated late in a primary immune response when the IL-2 secreted by antigen-activated T helper cells binds to high affinity IL-2 receptors expressed on antigen-activated B cells (Nakanishi et al., 1984; Matsui et al., 1989). The signal generated is then delivered to the promoter of the J chain gene, where it induces a nuclease hypersensitive region (Minie and Koshland, 1986) and activates synthesis of the J protein required for assembly of pentamer immunoglobulin M (IgM) antibody (Niles et al., 1995). Since these events can be reproduced in vitro in the IL-2-responsive BCL_1 cell line (Blackman et al., 1986; Brooks et al., 1983), expression of the J chain gene provides a useful model system for analyzing how cytokine-generated signals regulate genes involved in differentiative events.

The region of the J chain promoter that becomes hypersensitive upon IL-2 signaling extends approximately −170 bp upstream of the transcriptional start site. A major control sequence within this region has been defined by a combination of deletion and linked promoter analyses (Lansford et al., 1992; Shin and Koshland, 1993). Assays for loss of function in J chain–expressing plasmacytomas revealed two adjacent positive regulatory elements: a T-rich sequence (−70 to −57) denoted JA and a second purine-rich sequence (−56 to −45) denoted JB. The JB activity has been shown to be mediated by PU.1, a B cell– and macrophage-specific transcription factor belonging to the Ets oncoprotein family (Klemsz et al., 1990; Shin and Koshland, 1993). The factor responsible for JA function has yet to be positively identified. The contribution of each element was established by mutational analyses; base changes that prevented either NF-JA or PU.1 binding resulted in a 95% loss of promoter activity (Lansford et al., 1992; Shin and Koshland, 1993; S. Karray and M. E. K., unpublished data).

In the studies reported here, we used assays for gain of function in J chain–silent lymphomas to locate a negative regulatory element, JC, upstream of the JA and JB elements. Its activity was found to be mediated by the transcription factor, B cell lineage-specific activator protein (BSAP), a 50 kDa protein that binds DNA as a monomer (Adams et al., 1992). BSAP is a product of the *Pax5* gene, a member of a family of developmental control genes that encode transcription factors containing a paired DNA-binding domain. The nine mammalian *Pax* genes identified so far are transcribed primarily in the embryo and have been assigned important functions in the developing central nervous system based on their expression patterns and mutational phenotypes (Strachan and Read, 1994). In addition to its role in midbrain patterning, the *Pax5* gene is expressed in the B lymphoid lineage and has been shown to function throughout B cell development (Busslinger and Urbanek, 1995; Neurath et al., 1995b; Michaelson et al., 1996). Thus, targeted disruption of the mouse *Pax5* gene has established that BSAP expression is critical in early B lymphopoiesis (Urbanek et al., 1994). The mutant mice exhibited a block at a precursor stage and failed to produce pre-B, B, and plasma cells of the conventional or CD5 lineage. Moreover, BSAP-binding sites have been located in the promoter regions of a number of B cell–specific genes and shown by transient transfection analyses to activate transcription; these include the genes encoding the pre-B cell signaling molecules, $V_{pre\text{-}B}$ and λ5 (Okabe et al., 1992; Yang et al., 1995), as well as genes encoding molecules associated with antigen receptor signaling, the CD19 coreceptor (Kozmik et al., 1992; Kehrl et al., 1994), and the Blk Src kinase (Zwollo and Desiderio, 1994). Finally, in the antigen-driven stages of B cell differentiation, BSAP has been shown to up-regulate B cell proliferation (Wakatsuki et al., 1994), to be required for germline ε transcription that precedes an IgE switch (Liao et al., 1994), and to be relieved of its activity as a repressor of the 3′ heavy chain enhancer, 3′αE(hs1,2) (Singh and Birshtein, 1993; Neurath et al., 1994).

In this report, we identify BSAP as one of the factors responsible for the tight transcriptional control of the

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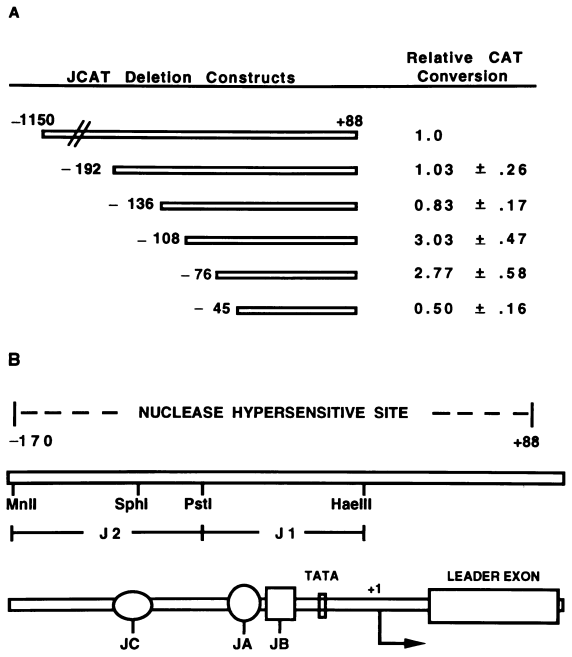

Figure 1. Effect of Deletions on the Promoter Activity of the J Chain Gene 5′ Flanking Region

(A) Identification of a negative regulatory element. Deletion constructs were transfected into J chain–silent PD31 pre-B cells and compared for CAT activity with the reference construct. Mean percentages of CAT conversion (±SEM) were calculated from the average values of at least three independent, duplicate determinations.
(B) Schematic diagram of the 5′ flanking region of the murine J chain gene showing the location of the negative regulatory element, JC, relative to the nuclease hypersensitive site, marker restriction enzyme sites, binding sites for other regulatory factors, and probes specific for JC and JA–JB sites.

immunoglobulin J chain gene. We present evidence that BSAP mediates the silencing of the J chain gene during the early stages of B cell development and is relieved of that function during the antigen-driven stage by, first, an IL-2-induced down-regulation of BSAP RNA expression and, second, the overriding effects of positive-acting factors, NF-JA and PU.1, binding to other promoter motifs. The demonstration that BSAP can block gene transcription directly by binding to an inherently repressive promoter site adds yet another function to its repertoire and thus strengthens its assignment as a key regulator of B cell differentiation.

Results

Identification of the JC Element by Deletion Analyses

The constructs used for deletion analyses of the J chain promoter have been described previously (Lansford et al., 1992). They contained a series of 5′ truncated sequences from the hypersensitive region (Figure 1A) that were inserted upstream of the chloramphenicol acetyltransferase (CAT) gene and a 1 kb sequence of heavy chain intron enhancer. To identify elements that repressed J chain gene transcription, these various constructs were transfected into the J chain–silent pre-B cell line PD31 and assayed for CAT activity (Figure 1A). The analyses showed that the intact J promoter was only minimally active in PD31 cells, the absolute percentage conversion of chloramphenicol to acetylated forms averaging .026 per microgram of protein per 0.1 mCi of [^{14}C]chloramphenicol, 0.4% of the activity of the same construct in the J chain–expressing S194 plasmacytoma cells (Lansford et al., 1992). Nevertheless, examination of the various deletion constructs indicated that a negative regulatory element, denoted JC, was located in the 5′ distal portion of the hypersensitive site, upstream of the already defined JA and JB motifs (Figure 1B). Thus, deletion of base pairs −136 to −108 produced a 3-fold increase in CAT activity over that of the intact promoter. Further deletion of the positive-acting JA and JB elements, located between base pairs −76 and −45, resulted in a 6-fold drop in CAT activity as previously reported (Lansford et al., 1992).

Characterization of a Nuclear Factor Interacting with the JC Repressor Element

Factor binding to the JC element was examined by gel mobility shift assays using a J2 probe encoding base pairs −168 to −84 (Figure 1B) and nuclear extracts from B cell and plasmacytoma lines representing successive stages in B cell development (Figure 2A). Of the several retained bands obtained, only the predominant, most rapidly migrating one exhibited a pattern consistent with our previous finding (McFadden and Koshland, 1991) that NF-JC binding to the JC element correlates inversely with J chain gene transcription (Figure 2A). Large amounts of complex were formed with extracts from J chain–silent cells, the PD31 pre-B line, the K46R mature B cell line, and the BCL_1 presecretor line. The amount was reduced approximately one half in extracts from BCL_1 cells induced by IL-2 to express J chain and further reduced in extracts from CH12LX cells that constitutively express low levels of J chain (Randall et al., 1992; Niles et al., 1995). Finally, the complex was just detectable in extracts from S194 plasmacytoma cells that contain high levels of J chain (Koshland, 1985). The gel mobility shift assays also indicated that NF-JC binding is B cell specific. As the binding pattern of extract from the T cell hybridoma BO_4H illustrates (Figure 2A), no complexes with the mobility or specificity of NF-JC were observed when nuclear extracts from the T cell lines EL-4 and CTLL, from the fibroblast line L929, and from human HeLa cells were incubated with the J2 probe (data not shown).

The JC element was first localized by gel mobility shift assays using J2 probes successively deleted at the 3′ end (Figure 2B). Removal of the 3′ 14 bp (−168 to −97) did not detectably alter NF-JC binding compared with that obtained with the wild-type J2 probe. However, removal of an additional 16 bp (−168 to −113) completely abrogated NF-JC complex formation, suggesting that base pairs −113 to −97 are essential for NF-JC binding. The results of the gel mobility shift assays were confirmed by methylation protection footprinting of the complex formed with the J2 probe (Figure 3A). The NF-JC-retained band gave a long, staggered footprint extending from base pair −126 through the SphI site (−118

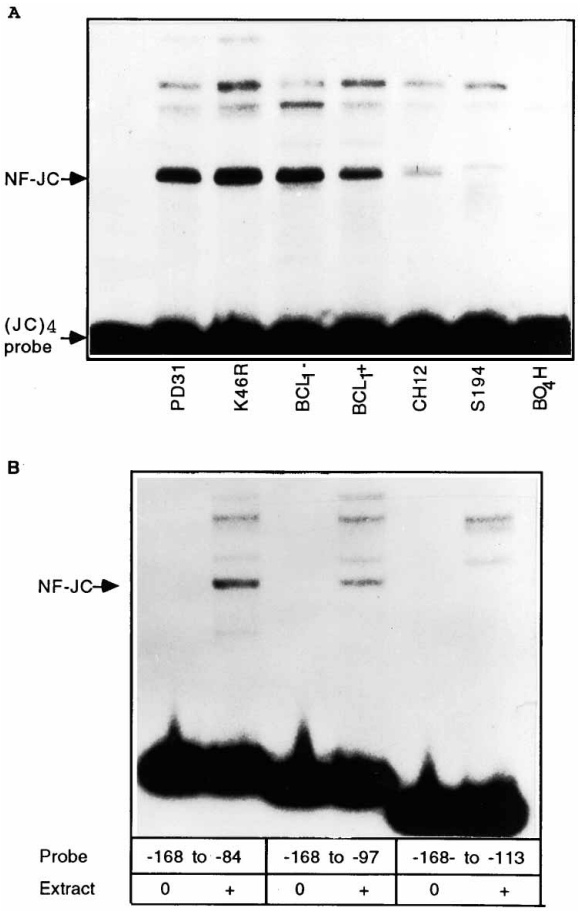

Figure 2. Gel Mobility Shift Analyses of the Binding of the Nuclear Factor, NF-JC, to the JC Element in the J Chain Gene Promoter

(A) NF-JC expression at successive stages of B cell development. For the binding reactions, labeled J2 probe was incubated either alone or with extracts from the PD31 pre-B cell line, the mature B cell line K46R, the BCL_1 presecretor line untreated or treated for 3 days with 100 U/ml of IL-2, the CH12LX presecretor line, the S194 plasmacytoma cell line, and the T cell hybridoma BO_4H.

(B) Localization of NF-JC-binding site. For the binding reactions, labeled J2 probes successively deleted at the 3′ end were incubated either alone or with nuclear extract from the mature B cell line K46R.

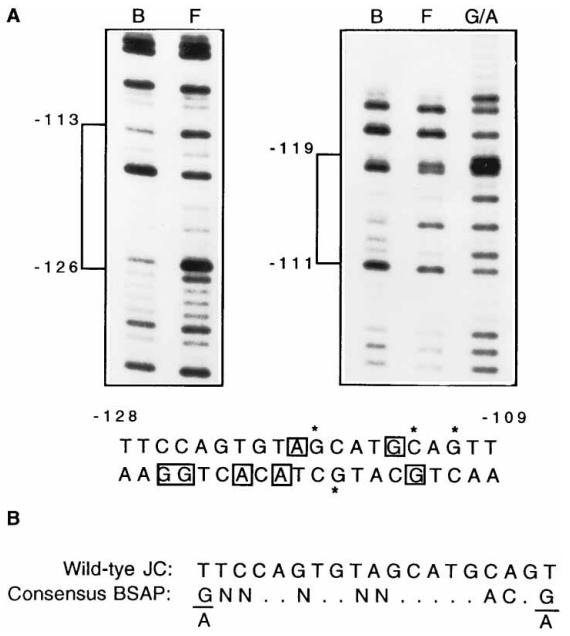

Figure 3. Dimethylsulfate Methylation Protection Analyses of NF-JC Binding

(A) Methylation protection patterns obtained on the coding strand (right) and noncoding strand (left) of the J2 promoter probe (−168 to −84) after reaction with nuclear extract from the J chain negative B cell lymphoma K46R. B, bound reaction; F, free reaction; G/A, cleavage at guanines and adenines. The NF-JC footprint is diagrammed below; boxes indicate protected residues, and asterisks indicate residues hypersensitive to cleavage.

(B) Comparison of the NF-JC footprint with the consensus BSAP recognition site determined by Czerny et al. (1993).

to −113) to base pair −111. The protected bases were located on the noncoding strand in the 5′ half of the footprint and on both strands in the 3′ half comprising the SphI site. In addition, nucleotides hypersensitive to methylation were located at positions −111, −113, and −118 on the coding strand and at position −117 on the noncoding strand.

Identification of NF-JC as the *Pax5* Gene Product, BSAP

A search of previously identified factor-binding sites in B lymphocyte genes revealed a considerable homology between the sequence recognized by NF-JC and those recognized by the BSAP product of the *Pax5* gene (Figure 3B). By aligning various BSAP-binding sites, Czerny et al. (1993) have defined a consensus sequence consisting of 5′ and 3′ half sites. Their studies have shown that two half sites are recognized by two subregions of the BSAP-paired DNA-binding domain and that the overall affinity of a given BSAP-binding site depends on the strength of interaction at each half site. This type of interaction allows for considerable variation in the sequences recognized by BSAP, since base changes in one half site that decrease binding affinity can be compensated by base changes in the other half site that increase binding affinity. As a result, none of the naturally occurring BSAP-binding sites, including the JC motif, completely conforms to the consensus sequence.

The homology in the sequences recognized by NF-JC and BSAP and their B cell–specific expression patterns suggested that the two factors might be identical. To obtain direct experimental evidence, we compared their DNA binding properties. Gel mobility shift assays showed that in vitro synthesized BSAP bound strongly to the JC oligonucleotide, and the resulting complex had the same mobility on a polyacrylamide gel as the complex formed with endogenously produced NF-JC protein (Figure 4A). Furthermore, both BSAP and NF-JC complexes were inhibited by a 100-fold molar excess of the classical BSAP recognition sequence from the histone H2A-2.2 gene promoter (Czerny et al., 1993), whereas neither complex was inhibited by a 100-fold molar excess of a mutant JC oligonucleotide, either JCM1 or JCM2, containing a 4 bp substitution that abrogated NF-JC binding (Figure 4B). Finally, gel mobility shift assays showed that rabbit antisera against the

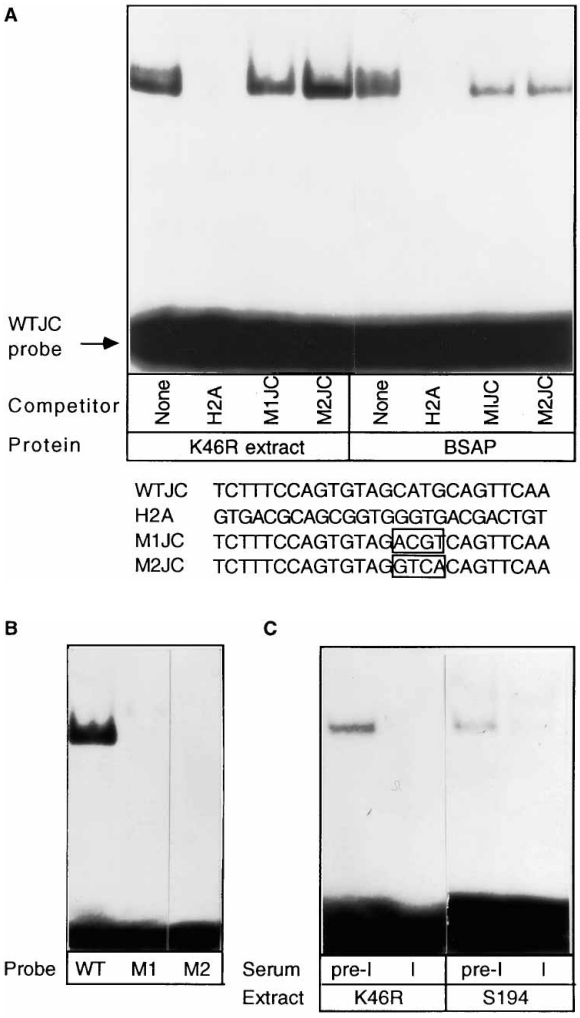

Figure 4. Identification of NF-JC as the BSAP Product of the *Pax5* Gene

(A) Comparison of the binding specificities of NF-JC and BSAP. Gel mobility shift assays of the binding of K46R nuclear extract and in vitro synthesized BSAP to the JC oligonucleotide in the absence or presence of a 100-fold molar excess of histone H2A-2.2 oligonucleotide (Czerny et al., 1993), M1 mutant JC oligonucleotide, or M2 mutant JC oligonucleotide. The sequences of the wild-type JC, the H2A 2.2 histone, and the mutant M1 and M2 JC oligomers are shown below; the substitutions made in the NF-JC-binding site are boxed.
(B) Effect of mutations on NF-JC binding. Gel mobility shift assays of the binding of K46R nuclear extract to the wild-type JC, the M1 mutant JC, and the M2 mutant JC oligonucleotides described in Figure 4A.
(C) Inhibition of NF-JC binding by anti-BSAP antibody. Gel mobility shift assays of the binding of K46R and S194 nuclear extracts to the JC oligonucleotide in the presence of preimmune serum (pre-I) or anti–human BSAP antiserum (I).

DNA-binding domain of human BSAP inhibited the recognition of the JC motif by the NF-JC protein endogenously produced by the mature B cell line, K46R, and by the plasmacytoma cell line, S194 (Figure 4C). The sum of these findings strongly indicated that NF-JC is BSAP, and it will be referred to as such hereafter.

Regulation of BSAP Expression by IL-2

Previous analyses of BCL_1 cells had shown that IL-2 treatment induces a progressive decrease in BSAP binding to the JC element in the J chain promoter (McFadden and Koshland, 1991). To determine whether the reduced binding reflected a decrease in BSAP synthesis, we extracted RNA from BCL_1 cells at intervals after IL-2 stimulation and determined the BSAP content by reverse transcription–polymerase chain reaction (RT–PCR). Two BSAP-specific products were observed (Figure 5) whether the RNA was extracted from untreated or IL-2-treated cells and whether the primers used generated full-length BSAP cDNA (Adams et al., 1992) or the amino-terminal two thirds. Comparison with size standards indicated that the more slowly migrating form had the size expected for a full-length cDNA, whereas the faster migrating form was approximately 150 bp shorter. Comparison of restriction enzyme digestion patterns located the base pairs missing from the shorter cDNA to the 5′ 290 bp of BSAP coding sequence. From the size and position of the deletion it seemed likely that the shorter cDNA species represented a BSAP isoform lacking exon 2 (base pairs 51 to 213) (Urbanek et al., 1994). Exon 2 encodes the amino-terminal half of the DNA-binding domain that interacts with the 3′ half site of the recognition motif (Czerny et al., 1993). Since mutations in the 3′ half site in the J chain promoter abrogate BSAP binding (Figures 4A and 4B), the shorter BSAP cDNA species was considered to encode a nonfunctional product, even if it were translated into protein.

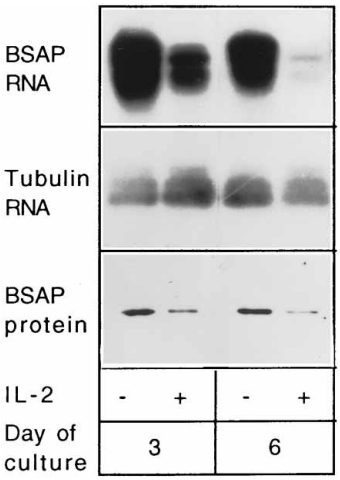

Figure 5. IL-2-Induced Decrease in BSAP RNA Levels

Top two frames, Southern blots of RT–PCR BSAP and tubulin cDNA from BCL_1 cells cultured in the presence and absence of 100 U/ml of IL-2. Bottom frame, Western blots of the BSAP content in nuclear extracts prepared from BCL_1 cells cultured as described above.

The longer, functional BSAP cDNA was quantitated by two different procedures. In one, the cDNA products were isolated by polyacrylamide gel electrophoresis and stained with SYBR green, and the dye uptake was measured by use of the fluorimager. In the second, the samples were analyzed by Southern blotting with a known BSAP cDNA fragment as probe, and the extent of hybridization was determined by use of a phosphorimager (Figure 5). The latter procedure also served to ascertain the specificity of the cDNA product. Tubulin cDNA was amplified and quantitated from each RNA preparation to correct for variability in RNA recovery and RT efficiency

Table 1. IL-2-Induced Down-Regulation of BSAP RNA and BSAP Protein Expression in BCL_1 Cells

Days after IL-2 Treatment	RNA Levels[a]	Protein Levels[b]	JC-Bound Protein[c]
Untreated[d]	100	100	100
3	60 ± 13[e]	55	56
6	23 ± 11	37	29

[a] BSAP RNA values were normalized to the tubulin content of the samples and expressed as a percentage of the values obtained from untreated cells at days 3 and 6.
[b] Western blot staining was quantitated by densitometer and expressed as described in (a).
[c] Data are taken from McFadden and Koshland (1991).
[d] Cultured for 3 or 6 days without IL-2.
[e] Standard error of the mean (SEM) calculated from at least three independent determinations.

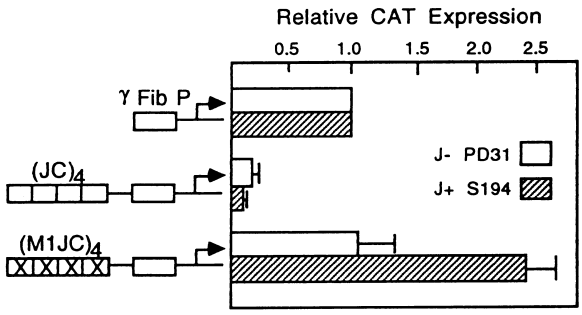

Figure 6. Linked Promoter Analyses of the JC Element

Constructs containing four copies of JC wild-type or mutant M1 oligomers were assayed for CAT activity in J chain–silent PD31 pre-B cells and in J chain–expressing S194 myeloma cells. CAT activity is expressed relative to the value obtained with the γ-fibrinogen promoter alone, which was set to 1.0. The values given represent the average of at least three independent duplicate determinations, and the calculated SEMs are represented by the error bars.

(Figure 5). The normalized BSAP values obtained showed that the IL-2 induces a down-regulation of BSAP RNA (Table 1). In BCL_1 cells, the level was reduced to 60% by day 3 and 23% by day 6 of IL-2 treatment. The IL-2-induced decrease in BSAP RNA content was reflected in the nuclear levels of BSAP protein, as determined by Western blotting (Figure 5), and paralleled the pattern of decrease previously observed in BSAP binding to the JC motif (Table 1).

Analyses of In Vivo BSAP Function

The deletion analyses of the J chain promoter hypersensitive site indicated that BSAP functions as a transcriptional repressor. Confirming evidence was obtained by examining the activity of the JC element in the context of a heterologous promoter. The construct used, pγ42 CassI, contained a truncated rat γ-fibrinogen promoter placed upstream of the CAT gene (Durand et al., 1987). Control experiments showed that this construct provided a low basal level of CAT expression in J chain–expressing myeloma lines, but not in J chain–silent B cell lines. An equivalent level was achieved in J chain–silent B cells, however, by inserting an immunoglobulin μ heavy chain enhancer sequence in 3′ to 5′ orientation downstream of the CAT gene.

For the JC functional assays, four copies of wild-type or mutant JC oligonucleotide were cloned into the pγ42CassI vector, and the resulting constructs were transfected into J chain–silent PD31 pre-B cells and J chain–expressing S194 myeloma cells. Analyses of CAT expression showed that the wild-type tetramer constructs were repressive in both cell types, reducing CAT synthesis 5-fold in PD31 cells and 9-fold in S194 cells (Figure 6). Moreover, the repression was shown to be BSAP specific in both cell lines. Thus, a tetramer construct containing the M1 4 bp substitution that abrogated BSAP binding (Figures 4A and 4B) had no repressive activity in PD31 cells and even induced a 2- to 3-fold increase in CAT activity in S194 cells.

BSAP Expression in Early and Late Plasma Cells

The finding that the JC tetramer construct was strongly repressive in the J chain–positive S194 cell line was surprising. It was not expected that BSAP would continue to function as a repressor in cells transcribing the J chain gene, particularly at the very low levels of the factor observed in S194 nuclear extracts (Figures 2A and 4B). To determine that the repressive activity observed was not an artifact of the oligomer constructs, we examined the function of a single JC element in the context of J chain promoter sequences. For these analyses, the following sequences were inserted into the pγ42CassI vector upstream of the minimal γ-fibrinogen promoter: the J2 fragment containing the JC element (−168 to −84); the J1 fragment containing the positive regulatory elements, JA and JB (−83 to −9); and a J1–J2 fragment (−168 to −9) containing all three elements.

Transfection of the J2 construct into J chain–expressing S194 cells confirmed the results obtained with the JC tetramers. The J2 fragment containing a single JC site was found to be strongly repressive, inducing a 9- to 10-fold decrease in CAT expression (Figure 7A). Two different criteria were used to ascertain that the repression was mediated by BSAP binding to the JC site. First, the M1 and M2 4 bp scrambles that were known to abrogate BSAP binding (Figure 4B) were introduced into the J2 fragment. Analyses of the resulting constructs showed that the mutations abolished the repressive activity of the J2 fragment, restoring CAT expression to that of the heterologous promoter control (Figure 7A). Second, the activity of the J2 construct was examined in MOPC315 cells, which do not express detectable amounts of BSAP (Neurath et al., 1994). Compared with its effect in S194 cells, the J2 fragment was only minorly repressive in MOPC315 cells (Figure 7A), reducing the transcriptional activity of the γ-fibrinogen promoter by one third. This small amount of repression may have reflected the activity of BSAP levels too low to be detected by gel mobility shift or RNase protection assays.

Transfection of the J1–J2 fragment into S194 cells, however, gave very different results; the J1–J2 fragment induced a 3-fold increase in CAT synthesis, compared with the 9- to 10-fold decrease induced by the J2 fragment alone (Figure 7B). Nevertheless, BSAP continued to bind to the J1–J2 fragment and exert some repression. The evidence was obtained by introducing the M1

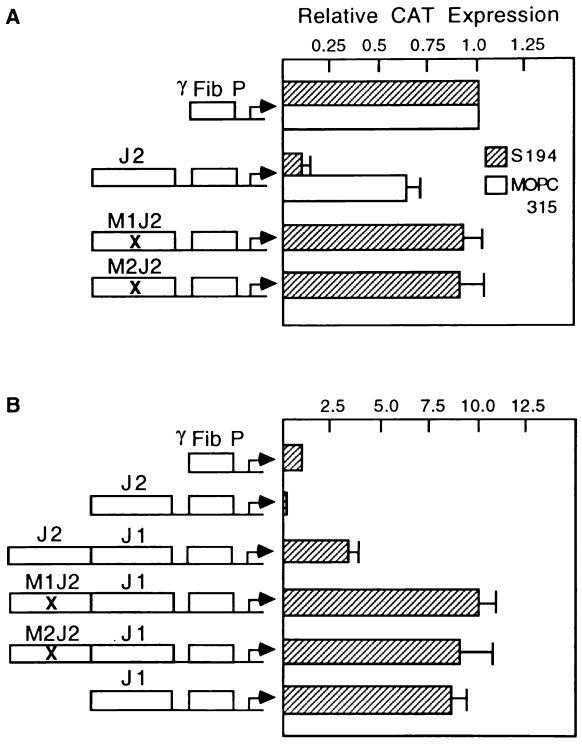

Figure 7. Linked Promoter Analyses of BSAP Function in J Chain–Expressing Plasmacytomas

(A) Repressive activity of the J2 promoter fragment in plasmacytomas. Constructs containing a single copy of the J2 fragment (−168 to −84) or the J2 fragment containing the M1 and M2 4 bp substitutions were assayed for CAT activity in J chain–expressing S194 and MOPC315 plasmacytoma cells.

(B) Overriding of JC repressive activity by downstream positive-acting elements. Constructs with single copies of the J2 promoter fragment (−168 to −84), the J1 promoter fragment (−83 to −9), the J1–J2 fragment (−168 to −9), or the J1–J2 fragment containing the 4 bp M1 and M2 substitutions were assayed for CAT activity following transfection into S194 cells. In (A) and (B), CAT activity is expressed relative to the value obtained with the γ-fibrinogen promoter alone, which was set to 1.0. All values are the mean of at least three independent duplicate determinations with the SEMs indicated by the error bars.

and M2 mutations into the JC site of J1–J2 sequence. The abrogation of BSAP binding resulted in an 8-fold increase in CAT activity, 2.5 times that achieved by the wild-type J1–J2 sequence and equivalent to that achieved by the J1 fragment alone (Figure 7B). Thus, BSAP was found to function as a repressor of J chain gene transcription even at the very low levels seen in S194 plasmacytoma cells, but in these cells the repression could be overridden by the positive activity of NF-JA and PU.1 factors bound to downstream J1 motifs.

Overexpression of BSAP in Plasma Cells

The results of the J1–J2 analyses suggested that BSAP concentration is a determining factor in the control of J chain gene expression. To test this hypothesis, we examined the effects of BSAP overexpression in S194 cells by both transient and stable transfection. Assays of transiently transfected BSAP activity were inconclusive. The BSAP expression vector was found to inhibit the CAT activity of cotransfected control plasmids, the pγ42CassI vector and a J promoter vector lacking the JC sequence, to almost the same extent as it inhibited the CAT activity of the J chain reporter constructs. Assays of stable BSAP transfectants, however, gave clear-cut results (Figure 8). Of 15 G418-resistant clones randomly selected for analysis, 11 were found to express recombinant BSAP transcripts, as judged by RT–PCR, and to synthesize BSAP factor, as judged by gel shift assays (data not shown). RNA was extracted from the cloned cells and analyzed on Northern blots by successive hybridization with J chain, BSAP, and tubulin probes. It was evident from inspection of the blots (Figure 8A) that the levels of J chain RNA were significantly lower in the BSAP transfectants than in untransfected S194 cells. Control experiments excluded the possibility that the decrease was effected by the expression vector itself (Figure 8B). In cells stably transfected with the BCMGSNeo vector or in BSAP transfectants that failed to express BSAP RNA, the levels of J chain RNA remained equivalent to that present in wild-type S194 cells. Lysates were also prepared from transfectant and control clones and examined for J chain protein by Western blotting. As expected, the levels of intracellular J chain were decreased in the BSAP transfectants to the same extent as the levels of J chain RNA (data not shown).

The extent of J chain RNA suppression was quantitated by scanning the membranes with a phosphorimager and correcting the J chain data for differences in tubulin content among the RNA preparations. The normalized values obtained (Figure 8A) showed that BSAP overexpression effected a 2- to 7-fold reduction in J chain RNA content. The degree of suppression in each transfectant was reproducible; for example, four independent assays of clones Tf4 and Tf5 gave J chain values relative to wild type of 0.13 and 0.27, with standard errors of the mean of 0.031 and 0.039, respectively. The different degrees of suppression among the transfectants correlated roughly with differences in BSAP RNA content; clones with a 4- to 7-fold decrease in J chain RNA contained approximately twice the levels of BSAP RNA as clones with a 1.5- to 2-fold decrease. The lack of a perfect correlation suggested that factors in addition to BSAP concentration, e.g., differences in the concentrations of NF-JA or PU.1, could influence the levels of J chain RNA in the individual clones.

The analyses of S194 cells overexpressing BSAP clarified several aspects of BSAP action. The observed down-regulation of J chain RNA provided direct in vivo evidence that BSAP functions as a repressor of J chain promoter activity. The fact that the down-regulation was achieved by overexpressing BSAP in cells actively synthesizing J chain RNA showed that BSAP repressor activity is concentration dependent. In view of these findings, it is likely that one function of the IL-2 signal delivered during a primary immune response is to reduce the BSAP concentration to a low enough level to allow the positive regulators of J chain gene expression to dominate.

Discussion

In the present study, the BSAP transcription factor was identified as a critical regulator of J chain gene expression. It mediates the silencing of the gene during the

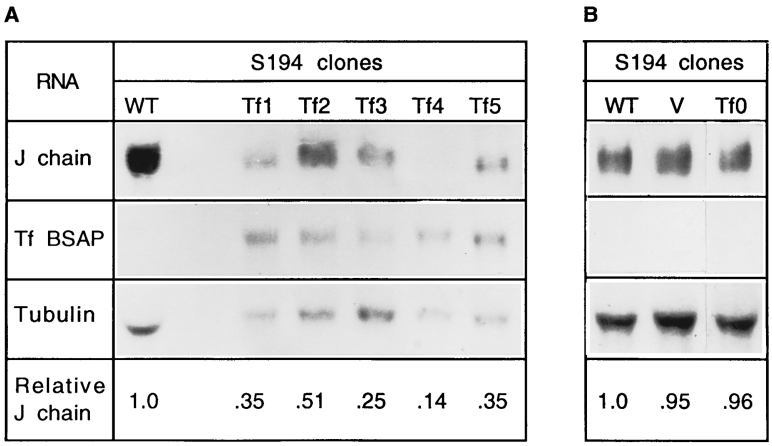

Figure 8. Effect of BSAP Overexpression in S194 Plasmacytoma Cells

Northern blot analyses of the J chain, transfected BSAP, and tubulin transcripts in (A) wild-type S194 cells and five randomly selected transfectants expressing recombinant BSAP and (B) wild-type S194 cells and representative clones of vector and nonexpressing BSAP transformants. The relative J chain RNA values were determined from phosphorimager measurements normalized to the tubulin RNA content. WT, wild type; Tf, transfected with BSAP expression vector; V, transfected with vector alone.

antigen-independent stages and the initial antigen-dependent stage of B cell development. The evidence for its silencing function is based on the following observations. First, BSAP expression correlates inversely with J chain expression. Second, BSAP binds specifically to the negative regulatory JC motif in the J chain gene promoter. Third, the JC motif functions as a transcriptional repressor only in those B cell lines expressing BSAP, and its repressive activity is relieved by mutations in the JC motif that abrogate BSAP binding. Finally, overexpression of BSAP in plasma cells represses high level transcription of the endogenous J chain gene.

The mechanism by which BSAP binding to the JC motif mediates repression of the J chain gene is not obvious. The JC recognition site is located approximately 100 bp upstream from the J chain TATA box and 50 bp away from the nearest positive regulatory element, the downstream JA motif. It seems unlikely that BSAP could function at this distance by any of the common repressor mechanisms, i.e., by competing with a positive regulatory factor for a similar or overlapping site (Harada et al., 1989; Jaynes and O'Farrell, 1988), by interfering with the DNA binding properties or activation function of a transcriptional activator (Benezra et al., 1990; Ron and Habener, 1992), or by directly preventing the formation of a transcription-competent RNA polymerase II complex (Gomez-Cuadrado et al., 1992; Inostroza et al., 1992). Bending of the J chain promoter DNA downstream of the JC element, however, could serve to bring BSAP within operating distance and allow it to function as a repressor, perhaps by interacting with a transcription complex subunit or by preventing access of NF-JA or PU.1 to their respective binding sites (Giese et al., 1992). Alternatively, BSAP could function from its upstream location by facilitating the stable positioning of a nucleosome over target positive regulatory motifs in the promoter (Roth et al., 1992). A blockage-type mechanism is supported by in vivo footprinting of the 3′ heavy chain enhancer (Neurath et al., 1995a). BSAP binding to its enhancer site was found to correlate inversely with the binding 50 bp downstream of an Ets-like factor that positively regulates enhancer activity. Interestingly, the J chain promoter has a similar arrangement of negative- and positive-acting motifs; the BSAP-binding site is located 50 bp upstream of the NF-JA and 60 bp upstream of the Ets PU.1-binding sites.

In addition to delineating a repressor function of BSAP, the present study demonstrated that the expression of BSAP is regulated by IL-2. The evidence was obtained by analyses of the IL-2-responsive presecretor line, BCL_1. IL-2 stimulation of these cells induced a progressive down-regulation in BSAP RNA levels that correlated with decreased BSAP binding to the JC repressor site and induction of J chain message (McFadden and Koshland, 1991; this paper). The BCL_1 system has also been used to identify a second IL-2-responsive factor, Blimp-1, which is a member of the zinc finger–containing family of DNA-binding proteins (Turner et al., 1994). Transcripts of the *Blimp-1* gene are rapidly induced by IL-2 or IL-5 treatment of BCL_1 cells (Turner et al., 1994) or by the addition of IL-2 and IL-5 to primary B cells treated with lipopolysaccharide (LPS) and anti–μ $F(ab')_2$ (Schliephake and Schimpl, 1996), and their expression leads to the induction of J chain message, high level IgM secretion, and other markers associated with early plasma cells. Similar changes can be induced by transfection of a *Blimp-1* expression vector into BCL_1 or naive B cells, suggesting that Blimp-1, like BSAP, is a key regulator in the differentiation of a mature B cell to an antibody secretor. Although BSAP and Blimp-1 have been identified as targets of IL-2 regulation, the mechanism by which IL-2 down-regulates BSAP expression on the one hand, and up-regulates Blimp-1 expression on the other, remains to be determined. The signal(s) could operate by controlling transcription initiation, transcript processing, RNA stabilization, or a combination of these mechanisms. In the case of BSAP, the finding of an isoform lacking a portion of the DNA-binding domain raises the possibility that down-regulation may be achieved by alternative processing.

The pattern of BSAP binding in cell lines representative of successive stages in B cell differentiation (Figure 2A) suggests that BSAP expression is constitutive until the early stages of an immune response. Then, a regulatory signal is initiated when the IL-2, and probably also the IL-5 (Matsui et al., 1989; McFadden and Koshland, 1991), secreted by antigen-activated T cells binds to high affinity IL-2 and IL-5 receptors induced on antigen-activated B cells. The signal(s) generated causes a progressive decrease in BSAP RNA accumulation that extends from the presecretor immunoblast through the plasma cell stages. Contrary to a previous report (Adams

et al., 1992) fully mature S194 plasmacytoma cells were found to maintain a low level of BSAP RNA, suggesting that BSAP may continue to be synthesized at a basal rate even at the terminal stage of B cell differentiation. Moreover, the small amount present in plasma cells remains fully functional, as judged in this report by measurements of its repressive activity.

The characterization of BSAP expression during antigen-driven B cell differentiation has considerable implications for understanding the dual regulatory functions of BSAP. IL-2-induced down-regulation of BSAP expression is consistent with a loss of function at BSAP positive regulatory sites, as is observed in the turning off of CD19 or Blk Src kinase transcripts. IL-2-induced down-regulation of BSAP expression is also consistent with a gain of function at negative regulatory sites, as is observed with BSAP release from the 3′ heavy chain enhancer and the J chain promoter sites. On the other hand, it is difficult to reconcile a decrease in BSAP expression with a gain in positive function, such as BSAP activation of B cell proliferation (Wakatsuki et al., 1994) and germline ϵ transcription (Liao et al., 1994). In particular, the germline ϵ transcription that precedes the switch to IgE synthesis is known to occur relatively late in a B cell primary response and to require an active 3′ heavy chain enhancer released from BSAP repression (Cogne et al., 1994).

The analyses of J chain gene activation presented here suggest that the different regulatory activities of BSAP may be accounted for by competition among the BSAP sites for the low levels of factor expressed at the early plasma cell stage. Thus, IL-2 may regulate BSAP concentration so that enough BSAP can bind to the LPS/IL-4-inducible Iϵ promoter to generate germline ϵ transcripts, but not enough interacts at the J chain promoter or 3′ heavy chain enhancer sites to block the dominating effects of positive regulatory factors binding downstream. This model is supported by the results of BSAP overexpression in plasma cells expressing high levels of J chain. By raising the BSAP concentration in these cells, it was possible to achieve enough BSAP binding to the J chain promoter to reverse the positive activity of NF-JA and PU.1 and thus significantly reduce the expression of the J chain gene.

Experimental Procedures

Cell Culture

The cell lines representative of pre-B, immature B, and mature B stages of development were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U of penicillin per milliliter, and 100 μg of streptomycin per milliliter. The various myeloma and hybridoma lines were cultured in Dulbecco's modified Eagle's medium supplemented as described above.

Preparation and Assay of Nuclear Proteins

Large-scale extracts were prepared from 10^9 cells by the detergent lysis method of Peterson et al. (1986) as modified by Lansford et al. (1992). All buffers contained the following cocktail of protease inhibitors: 0.5 nM phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol, 0.5 mM $Na_2S_2O_5$, aprotinin (10 U/ml), leupeptin (5 mg/ml), and pepstatin A (5 mg/ml). Mini-extracts were prepared from 10^7 cells as described previously (McFadden and Koshland, 1991). To obtain BSAP protein for analysis, BSAP cDNA was isolated from the BSAP-BCMGSNeo plasmid by XhoI–NotI cleavage, the 5′ end was blunt ended, and the resulting fragment was cloned into the pcDNAI (Invitrogen) polylinker by use of a blunt-ended HindIII and a NotI site. The plasmid was linearized with XbaI, transcribed in vitro with T7 RNA polymerase, and translated in vitro with a rabbit reticulocyte lysate (Promega) according to the protocols suggested by the manufacturer.

For gel mobility shift assays, the specific oligonucleotides were end labeled with [α-^{32}P]dCTP and Klenow enzyme (Sambrook et al., 1989). The binding reactions with crude nuclear extracts were performed as described previously (McFadden and Koshland, 1991) using 8–10 μg of extract, 4–6 μg of poly(dI–dC) nonspecific competitor, and 10^4 cpm (0.1–1.0 ng) of probe. The binding reaction with in vitro synthesized BSAP used 5 μl of reticulocyte lysate and 4 μg of nonspecific competitor. For the antibody blocking assays, crude nuclear extracts were preincubated with 2 μl of a 1:10 dilution of either rabbit preimmune sera or rabbit anti–human BSAP antiserum directed against the DNA-binding paired domain. In each case the protein–DNA complexes formed were resolved from free probe by electrophoresis through glycerol-containing 5% polyacrylamide gels (29:1) containing 0.25× TBE buffer.

Footprinting Assays

Methylation protection footprinting was performed with crude nuclear extracts from the mature B cell line, K46R; 50 μg of nuclear protein was incubated for 15 min at 0°C with 2 μg of poly(dI–dC) and 10^6 cpm of DNA probe. Dimethylsulfate (0.5 μl) was added for 45 s and quenched by the addition of dithiothreitol to a final concentration of 23 mM. The remaining steps in the assay, isolation and sequencing of free and protein-bound DNA, were performed according to the standard protocol for methylation interference footprinting (Ausubel et al., 1987). The probe was a 100 bp XbaI–HindIII fragment containing the J chain sequence −168 to −84 that was end labeled on the top or bottom strand.

Plasmid Constructions for Linked Promoter Analyses

In the pγ42CassI vector (Durand et al., 1987) the CAT gene is under control of a truncated γ-fibrinogen promoter (−54 to +36) that includes a TATA box and a single Sp1-binding site. Fragments from the J chain promoter (J1, base pairs −83 to −9; J2, base pairs −168 to −84; J1–J2, base pairs −168 to −9) and oligonucleotides representing the JC element and mutant JC sequences were synthesized with XbaI linkers and inserted either singly or in multiple copies into the polylinker upstream of the γ-fibrinogen promoter. All constructs were sequenced to determine oligomer copy number and orientation.

Mutagenesis of the pγ42CassI plasmid containing either the J2 or the J1–J2 sequence was performed with the Transformer site-directed mutagenesis kit (Clontech Laboratories). Two primer sequences, 5′-CTTTCCAGTGTAGACGTCAGTTCAAACCTG-3′ and 5′-CTTTCCAGTGTAGGTCACAGTTCAAACCTG-3′, were used to introduce each 4 bp change in the JC site (JCM1 and JCM2 mutants, respectively), whereas a second primer sequence, 5′-GCCCCCGTT TTCACGATGGGCAAATATTA-3′, was used to introduce a single base pair change in the NcoI site in the CAT gene. The latter change eliminated cleavage by NcoI without altering the CAT amino acid sequence and thus provided a ready screen for mutant constructs. The selected plasmid was sequenced to verify the base substitutions.

Transfections

Transient transfections of S194 myeloma cells and PD31 pre-B cells were performed by the DEAE–dextran technique (Grosschedl and Baltimore, 1985) and transfections of MOPC315 myeloma cells by electroporation (Neurath et al., 1994). In each case, 10^7 cells in logarithmic growth phase were transfected with 9 μg of supercoiled test plasmid or a combination of plasmids. Cell extracts were prepared 44–48 hr after transfection and assayed for CAT activity (Lansford et al., 1992).

For the generation of stable transfectants, 8 μg of the BSAP-BCMGSNeo construct (Neurath et al., 1994) or the BCMGSNeo vector were introduced into S194 cells by electroporation; 1.5 × 10^7

cells suspended in 270 μl of RPMI medium were transfected at 250 V and 960 μF capacitance using a Gene Pulsar (Bio-Rad). Samples (100 μl) of the transfected cells were aliquoted into 96-well plates and 24 hr later mixed with an equal volume of 2× G418–sulfate selection medium. G418-resistant clones were detectable by microscopy after 2 to 3 weeks of culture. At that time, cells from positive wells were replated at a concentration of less than 1 cell per well, expanded, and maintained in G418 selection media.

RNA Isolation and RT–PCR

Total RNA was isolated from populations (5×10^6 cells) of unstimulated and IL-2-stimulated BCL_1 cells with RNASTAT-60 buffer (TEL-TEST). RNA aliquots of 10 μg were transcribed into cDNA with AMV reverse transcriptase and specific BSAP and tubulin transcripts were amplified by use of the following primer pairs: BSAP1, 5′-ATCAGGACAGGACATGGAGGAGTG-3′ and 5′-TGGTCTGTTCT GGCTTGATGGG-3′; BSAP2, 5′-GCGAGATCTCCATTTCATCAAG TCCTGAAA-3′ and 5′-GCGAAGCTTGGTCARTGNCGRTCRTANGC NGT-3′; tubulin, 5′-CAGGCTGGTCAATGTGGCAACCAGATCGGT-3′ and 5′-GGCGCCCTCTGTTAGTGGCCTTTGGGCCCA-3′. The BSAP PCR reactions were run for 29–31 cycles at 94°C for 30 s, at 59°C for 1 min, and at 72°C for 2 min; for tubulin, the reactions were carried out for 27 cycles at 94°C for 30 s, at 50°C for 1 min, and at 72°C for 1 min. The number of cycles used for each primer set fell within the linear range of cDNA production. BSAP and tubulin products were measured by two methods. For Southern blots, the PCR samples were fractionated on agarose gels, transferred to nitrocellulose and probed with a 582 bp BSAP cDNA fragment or with a 310 bp tubulin cDNA fragment random primer labeled with both [α-^{32}P]dCTP and [α-^{32}P]dATP. The extent of hybridization was then quantitated by use of the phosphorimager. For the second method, the PCR products were run on polyacrylamide gels and stained with a 1:10,000 dilution of SYBR green (Molecular Probes), and the dye uptake was quantitated with a fluorimager (Molecular Dynamics). The scanning data from the phosphorimager and fluorimager were analyzed by an ImageQuant program.

Northern Blot Analyses

Northern blots were performed with 20 μg of total RNA as described by Lamson and Koshland (1984). RNA was size fractionated by electrophoresis on a 1.4% formaldehyde–agarose gel, transferred to a Zeta probe nylon membrane (Bio-Rad), and hybridized with [α-^{32}P]dCTP-labeled J chain cDNA (Niles et al., 1995), a 532 bp BSAP cDNA fragment, and a cDNA fragment of rat tubulin (gift of A. Winoto).

Western Blots

Nuclear extracts (25 μg) from IL-2-stimulated and unstimulated BCL_1 cells were boiled for 5 min, size fractionated by SDS–polyacrylamide gel electrophoresis (8%), and transferred to a nitrocellulose filter. After pretreatment with 1% dry milk in 1× PBS, the filters were incubated for 3 hr with a 1:10,000 dilution of antibody specific for the BSAP paired domain. They were then washed and incubated for 1 hr with 1:5,000 dilution of horseradish peroxidase–conjugated goat anti–rabbit IgG. After several washes, the filters were developed using enhanced chemiluminescence (Amersham).

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